Report as of FY2011 for 2011PA158B: "Fish Exposure to Emerging Contaminants in Municipal Wastewater: Can Dietary Sewage Contribution Predict Severity of Estrogenic Effects?"

Publications

Project 2011PA158B has resulted in no reported publications as of FY2011.

Report Follows

Pennsylvania Water Resources Research Center
FY2011 Final Report

PROJECT TITLE AND PRINCIPAL INVESTIGATORS

Fish exposure to emerging contaminants in municipal wastewater: can dietary sewage contribution predict severity of estrogenic effects?

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KEYWORDS: emerging contaminants, pharmaceuticals, endocrine disruption, estrogenic effects, intersexuality, reproductive anomalies in fish, municipal wastewater, stable isotopes, white sucker, *Catostomus commersoni*

STATEMENT OF WATER PROBLEM

A number of organic contaminants, including pharmaceuticals, personal care products, plasticizers, pesticides and herbicides, and their breakdown products, have been shown to have endocrine activity and the potential for endocrine disruption in a range of vertebrates, even at very low concentrations (Sumpter and Johnson 2005; Vajda et al. 2007). Fish appear to be particularly vulnerable due to their constant environmental exposure, particularly in the presence of effluent from wastewater treatment plants (Sumpter and Johnson 2005). Effects include altered sex ratios (e.g., Vajda et al. 2007) and high proportions of intersex fish (generally apparent males with testicular oocytes; Blazer et al. 2007, Vajda et al. 2007).

In 2006, the United States Geological Survey (USGS) surveyed a number of small streams in south-central Pennsylvania for pharmaceuticals and antibiotics at sites upstream and downstream of wastewater treatment plants (WWTPs) and animal-feeding operations. This survey, part of a larger effort in cooperation with the Pennsylvania Department of Environmental Protection (DEP), sought to document concentrations of a number of emerging contaminants in both streamwater and wellwater across Pennsylvania (Loper et al. 2007). Thirteen pharmaceuticals and eleven antibiotics were detected at least once in the survey; stream sites receiving municipal wastewater had the highest number of detections and highest concentrations of detected contaminants. Although this survey was not designed to specifically assess the potential for endocrine disruption, several of the detected pharmaceuticals have potential estrogenic effects (Loper et al. 2007), and the presence of so many detectable pharmaceuticals suggests others more directly linked to endocrine disruption are likely present as well.

The objectives of this study are to examine fish (probably white sucker, *Catostomus commersoni)* in several of the same streams surveyed by USGS for reproductive anomalies that could be indicative of exposure to endocrine disrupting contaminants. We also plan to assess dietary exposure to sewage-derived organic matter using stable isotopes of carbon and nitrogen, and to determine whether these two potential indicators of contaminant exposure

are correlated with one another. Finally, we will examine the operations of each WWTP in the study and relate key aspects of their operation, as well as degree of dilution of effluent in the receiving stream (Sumpter and Johnson 2005), with the effects detected in fish.

NATURE, SCOPE, AND OBJECTIVES

This study will involve field and lab research in three small wastewater-influenced streams in south-central Pennsylvania to determine the frequency and severity of reproductive anomalies in fish, as well as the proportion of fish diets assignable to sewage-derived organic matter. Specific objectives:

(1) Determine the sex ratio of white sucker in Middle Spring, Mountain Creek, and Killinger Creek upstream and downstream of wastewater effluent sources.
(2) Determine the presence and severity of intersexuality in white sucker in all three streams upstream and downstream of effluent sources.
(3) Using stable isotopes of carbon and nitrogen, determine the proportion of fish diet that can be ascribed to sewage-derived C and N, and relate that to the incidence and severity of reproductive anomalies.
(4) Determine the proportion of stream flow attributable to the effluent source in each season and relate to the incidence and severity of reproductive anomalies.

METHODS, PROCEDURES, AND FACILITIES.

Sample sites will be selected upstream and downstream of effluent release points in three south-central Pennsylvania streams: Middle Spring and Mountain Creek in Cumberland County, and Killinger Creek in Lebanon County. All three are relatively small (2^{nd}-4^{th} order) streams in the lower Susquehanna River drainage in which treated wastewater makes up a significant portion of discharge at downstream sites. Of the wastewater-affected south-central PA sites sampled for pharmaceuticals by the USGS, these represent accessible streams which have a range of detection levels, from low (Mountain Creek) to high (Killinger Creek). We will make collections three times: in spring (April/May), summer (July/August) and fall (September/October) to detect any seasonal variability in effluent effects and fish reproductive condition. Basic physical (average width and depth, velocity, discharge, temperature) and chemical (pH, specific conductance, dissolved oxygen) data will be recorded at each site using meters owned by Shippensburg University. Conductance will also be tested in or as close as possible to the effluent release point for use in determining the proportion of downstream discharge attributable to wastewater effluent using a two-source mixing model (Lentek-Klemunes and Hurd, submitted). This determination will be compared to one based on direct measures of discharge upstream and downstream of the WWTP.

Fish will be sampled by single-pass electrofishing, using an Appalachian Aquatics backpack electroshocker (Model AA-24) owned by Shippensburg University. Our expectation is that we will focus on white sucker *(Catostomus commersoni)* as they are common in most small streams (including degraded sites) in our area and have been used successfully in studies of effluent effects on sex ratios and intersexuality in other studies (e.g., Vajda et al. 2007). However, since one source (V. Blazer, pers. comm.) suggests they may not be particularly sensitive to environmental estrogens, we will make a final determination of focal species after examining a test collection of fish collected from the

Middle Spring effluent site in fall 2010. Other abundant species in Middle Spring and Mountain Creek which could potentially be substituted include various minnows (Cyprinidae) and sculpins (*Cottus* sp.). Centrarchids such as smallmouth bass, the focus of much of Dr. Blazer's recent work (e.g., Blazer et al. 2007), are not abundant in these streams.

Fish for gonad examination and stable isotope analysis (20 per site on each collection date) will be anaesthetized in the field using clove oil and ethanol, then killed with a blow to the head. They will be returned to the lab on ice, measured (total and standard length) and weighed for calculation of condition index and correlation with other measures. An initial sex determination will be made based on gonad appearance; the gonads will then be removed from the fish and embedded in paraffin. The tissues will be sliced with a microtome and stained with hematoxylin and eosin (H and E). Gonads will be sliced in 5 cross sections from different regions and examined using a light microscope. The severity of intersexuality will be graded on a 0-4 scale using methods described by Blazer et al. (2007). A small sample of tail muscle will be saved from each fish and frozen (-20ºC) for later preparation for isotope analysis. All samples, slides, and photographs will be coded with a fish identification number associated with all measures for that individual to allow for later statistical analyses.

Muscle tissue and for stable isotope analysis will be dried at 55-60ºC for 24-48 hours, then ground and homogenized using a mortar and pestle. Samples will be shipped to the Cornell Stable Isotope Laboratory, where they will be analyzed for $\delta^{13}C$ and $\delta^{15}N$ with a Europa Scientific GEO 20-20 isotope ratio mass spectrometer. Additionally, we will obtain samples of sewage-derived particulate organic matter (SDPOM) either from WWTP personnel at each site (from the blanket particulate layer in the final clarifier) or by filtering effluent water through a pre-combusted glass filter. These samples will be air-dried, then prepared as above for stable isotope analysis and shipped to Cornell for analysis.

We will calculate the relative contribution of sewage-derived organic matter (SDPOM) in white sucker diets by measuring isotopic signatures of consumers at downstream sewage-exposed sites and comparing them to that of the sewage and of upstream white sucker (deBruyn & Rasmussen 2002). This approach uses a two-source, single tracer mixing model to determine relative contribution of the sources to a common sink (Eq. 1);

$$1.0\ (\delta R_{downstream}) = X\ (\delta R_{SDPOM} + b) + (1\text{-}X)\ (\delta R_{upstream} + b) \qquad \text{Eq. 1}$$

where δR is the relative stable isotope abundance in fish below outfall ($\delta R_{downstream}$), in sewage derived particulate organic matter (δR_{SDPOM}), and in fish from the upstream reference area ($\delta R_{upstream}$); b is the trophic step factor; and X is the proportion of sewage-derived element R in fish. We will assume a trophic shift of 0 for $\delta^{13}C$ and 3 for $\delta^{15}N$ (deBruyn & Rasmussen 2002).

PRINCIPAL FINDINGS AND SIGNIFICANCE

Emerging contaminants in Pennsylvania waters include pharmaceuticals, personal care products, and other common chemicals with potential endocrine activity. Recent concern about these contaminants has focused on their presence in treated wastewater and effects on fish, which may be particularly vulnerable due to their constant environmental exposure. Affected populations may show altered sex ratios or the presence of intersex (usually testicular oocytes [TO]). A recent USGS survey (Loper et al. 2007) examined a

number of small streams in south-central Pennsylvania for pharmaceuticals and antibiotics at sites upstream and downstream of wastewater treatment plants (WWTPs). Thirteen pharmaceuticals and 11 antibiotics were detected at least once in the survey; stream sites receiving municipal wastewater had the greatest number of detections and highest concentrations of detected contaminants.

The objectives of this study were to examine fish in three of the same streams surveyed by the USGS for reproductive anomalies that could indicate exposure to endocrine disrupting contaminants. We also assessed dietary exposure to sewage-derived organic matter using stable isotopes of carbon and nitrogen, and attempted to determine whether these two potential indicators of contaminant exposure were associated with one another. Stable isotope signatures also served as an indicator of site fidelity. A distinct shift in ratios of heavy to light stable isotopes of carbon and nitrogen (in the direction of greater enrichment of heavy isotopes) is commonly seen in organisms living downstream of sewage effluent. Finding that shift in downstream fish (especially any intersex fish) would demonstrate they had indeed been exposed to sewage effluent. We hypothesized that (1) we would find stable isotope shifts in downstream fish indicating some dietary sewage exposure, (2) if we found intersex fish, they would be found only downstream of WWTPs, and would have stable isotope signatures indicating sewage exposure, and (3) intersex fish would have isotope ratios indicating *greater* dietary sewage exposure than other downstream fish.

We initially collected fish of three species (white sucker *Catostomus commersoni*, mottled sculpin *Cottus bairdi*, and eastern blacknose dace *Rhinichthys atratulus).* However, only blacknose dace were found consistently in all streams and sites and (usually) in sufficient numbers to allow us to collect 20 adults at each site and sampling occasion, so they became our focal species. We collected dace upstream and downstream of municipal wastewater treatment plants in Middle Spring and Mountain Creek (both of Cumberland County) and Quittapahilla Creek (Lebanon County). Temperature, pH, and specific conductance were measured at each site. Up to twenty sexually mature dace were collected at each site in May, July, and October 2011 using a backpack electrofisher. Fish were euthanized (using a dilute solution of clove oil and ethanol in water) on site and transported back to the lab on ice. In the lab, gonads of all male fish and most females were removed and prepared for histology using standard methods, then sliced, stained, and examined for abnormalities on a compound microscope at 100x. A portion of each fish tail was frozen and later dried and prepared for stable isotope analysis, which was carried out at the Cornell Stable Isotope Laboratory. Carbon and nitrogen stable isotope ratios for downstream fish were compared to those of upstream fish and organic matter from the blanket layer of the final clarifying tank at each of the WWTPs to estimate the proportion of sewage derived carbon and nitrogen in the diets of downstream fish.

Stable isotope analysis indicated dietary sewage uptake by blacknose dace in all three streams, with the greatest enrichment in the Quittapahilla Creek and least in Mountain Creek. All three creeks showed significant enrichment of both carbon and nitrogen at all sampling dates, with the exception of carbon in Middle Spring in fall. We estimated that a mean of about 41±5% (±1 SE) of dietary carbon was derived from sewage in the Quittapahilla, and 23±12% in Middle Spring. Estimates for Mountain Creek, where both upstream and downstream values were close to the sewage value, were too variable to estimate with any confidence. Blacknose dace, which have a diet of about 25% algae and 75% aquatic

invertebrates, are probably gaining most of this sewage-derived carbon second-hand, by feeding on invertebrates that consume detritus directly. Filter-feeding and deposit feeding invertebrates in Middle Spring just below the WWTP have previously been shown to derive up to nearly 80% of their carbon from sewage sources (Lentek-Klemunes 2008).

We found evidence of intersex (TO) in three male fish, all downstream of WWTP discharges, but no evidence of altered sex ratios in downstream fish. One intersex dace was collected in summer in the Quittapahilla and two in Middle Spring, one in spring and one in fall. Intersex in these three fish was confirmed by Vicki Blazer of the USGS Leestown Science Center, Fish Health Branch. This was consistent with our hypothesis, but the association of intersex with downstream collection site was only marginally significant (Fisher's exact test, $P = 0.093$). This is, to our knowledge, the first documentation of intersex in blacknose dace. Since it is a common and often abundant species in eastern streams and smaller rivers, is tolerant of degraded conditions, and is easy to collect by seining or electrofishing, it has the potential to serve as an indicator of xenoestrogen contamination in smaller streams where known estrogen-sensitive species such as black basses may rarely occur.

With only three intersex fish, we don't have strong evidence for or against our third hypothesis. One of those fish (collected in fall in Middle Spring) did have a significantly more enriched carbon signature than the rest of the downstream fish from that site and collection date ($t_{12} = -3.58$, $P = 0.004$), but the other two did not, showing values close to the mean downstream value in both cases. Intersex in gonochoristic fish is probably a result of a combination of genetic sensitivity to estrogen and timing of exposure, with some studies indicating a narrow window of sensitivity in early development, so these more stochastic factors are likely more important than overall exposure in determining which fish are affected to a detectable degree.

With the extension of our project into this year, we plan to focus further collection on Middle Spring, and perhaps Three Square Hollow Run in Cumberland County, which was surveyed by Loper et al. (2007) and is affected by runoff from a confined agricultural feeding operation. We will collect more blacknose dace immediately downstream of the Shippensburg WWTP, but also add collection points further downstream to see how far downstream intersex can be detected. We also plan to directly examine stomach contents of selected fish to more accurately estimate the trophic position of dace in the food web.

LITERATURE CITED

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Spies, RB, H. Kruger, R. Ireland, and D.W. Rice, Jr. 1989. Stable isotope ratios and contaminant concentrations in a sewage-distorted food web. Marine ecology progress series 54(1-2): 157-170.

Sumpter, JP and AC Johnson. 2005. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environmental Science and Technology 39(12): 4321-4332.

STUDENTS & POSTDOCS SUPPORTED

Lauren E. Kesslak, Biology, MS expected May 2012
Tammy Smith, Biology, MS student (volunteer, continuing)
Pat Bower, Biology, MS student (volunteer, continuing)

PUBLICATIONS

Kesslak, Lauren E. 2012. *The effects of wastewater exposure on blacknose dace (Rhinichthys atratulus) in South-Central Pennsylvania*, MS Thesis, Department of Biology, Shippensburg University, Shippensburg, PA, 48 pp.

PRESENTATIONS

Kesslak, Lauren E., Tammy Smith, and Theo Light. Fish exposure to emerging contaminants in municipal wastewater: can dietary sewage contribution predict severity of estrogenic effects? Presented at the 2012 annual meeting of the Pennsylvania Academy of Science, Allentown, PA, March 31.

OTHER INFORMATION TRANSFER ACTIVITIES
Either Lauren or Theo will present our results at the Middle Spring Watershed Association's May, 2012 meeting.

NOTABLE AWARDS & ACHEIVEMENTS
Lauren Kesslak was recently accepted to the Ph.D. program in Fisheries at West Virginia University, with a research assistantship to support continuing work on the effects of emerging contaminants on fish health.

ADDITIONAL FUNDING ACQUIRED USING USGS GRANT AS SEED MONEY
SU College of Arts and Sciences Faculty-Led Research Fund, $570. April 2011-June 2012. Fish exposure to emerging contaminants in municipal wastewater: can dietary sewage contribution predict severity of estrogenic effects?

PHOTOS OF PROJECT

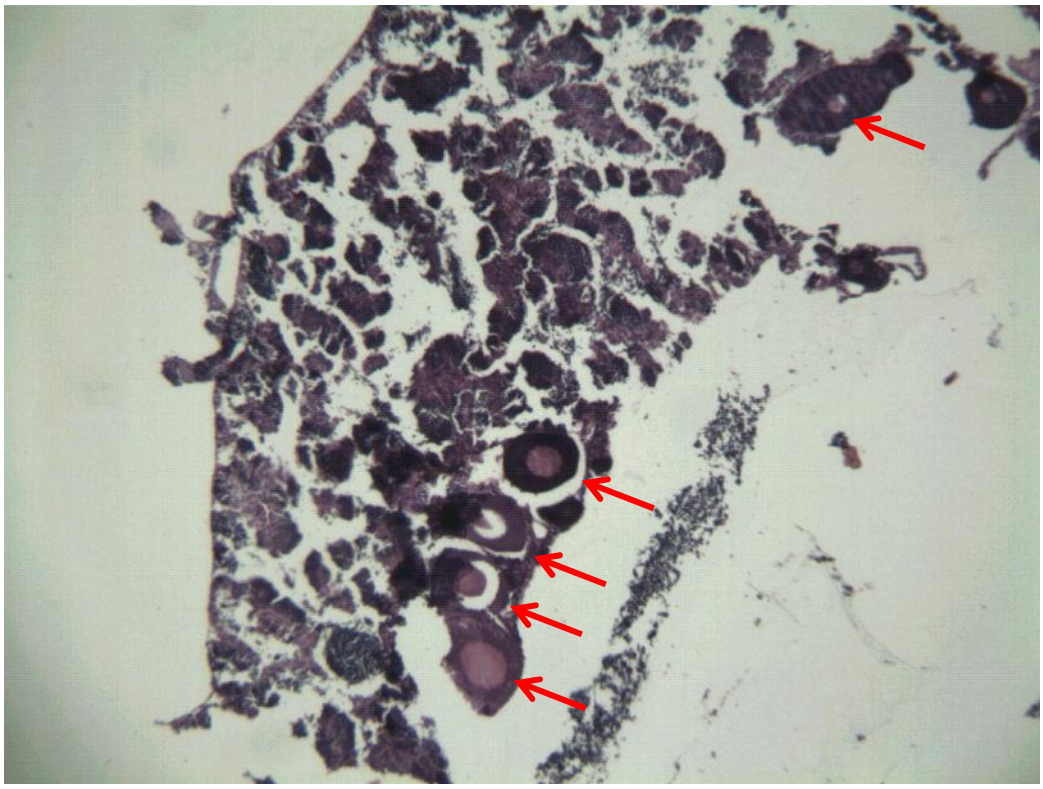

Figure 1: Gonad of an intersex blacknose dace from Middle Spring, showing primary oocytes (red arrows) in the testis of a male-appearing fish.

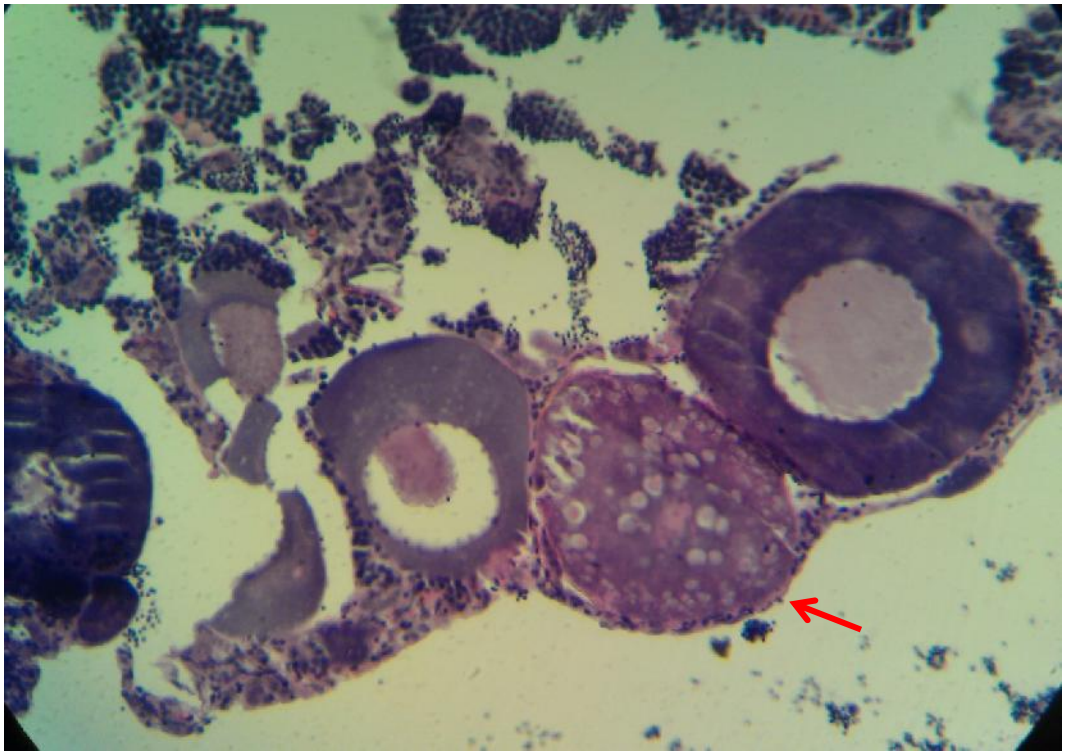

Figure 2: Gonad of an intersex blacknose dace from Middle Spring, showing primary and secondary oocytes (red arrow) in the testis of a male-appearing fish.

Figure 3: Electrofishing in Middle Spring Creek at Bard Road, Shippensburg. (L => R: Lauren Kesslak, Theo Light, and Tammy Smith)